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Altered Regulation of Gene and Protein Expression of Hypothalamic-Pituitary-Adrenal Axis Components in an Immature Rat Model of Chronic Stress

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Abstract

Chronic stress early in postnatal life influences hormonal and behavioural responses to stress persistently, but the mechanisms and molecular cascades that are involved in this process have not been clarified. To approach these issues, a chronic stress paradigm for the neonatal rat, using limited bedding material to alter the cage environment, was devised. In 9-day-old rats subjected to this chronic stress for 1 week, significant and striking changes in the expression and release patterns of key molecules that govern the neuroendocrine stress responses were observed. The presence of sustained stress was evident from enhanced activation of peripheral elements of the neuroendocrine stress response, i.e. increased basal plasma corticosterone concentrations, high adrenal weight and decreased body weight. Central regulatory elements of the neuroendocrine stress response were perturbed, including reduced expression of hypothalamic corticotropin-releasing hormone that, surprisingly, was accompanied by reduced glucocorticoid receptor expression. Thus, the effects of chronic sustained stress in the neonatal rat on the hypothalamic-pituitary-adrenal axis included substantial changes in the expression and activity of major regulators of this axis. Importantly, the changes induced by this chronic stress differed substantially from those related to acute or recurrent stress, providing a novel model for studying the long-term effects of chronic, early life stress on neuroendocrine functions throughout life.

Keywords

corticotropin releasing hormone; chronic stress; neonatal rat; CRF; glucocorticoid receptor

In both humans and experimental animals, stress early in life may lead to affective and anxiety disorders (1, 2). These are often associated with dysfunctional hypothalamic-pituitary-adrenal (HPA) axis, involving corticotropin releasing hormone (CRH) as well as glucocorticoids and their receptors (GRs) (2, 3). The mechanisms by which early life stress provokes these effects are not well understood, but may involve molecular signalling processes that are induced by stressful challenges in the immature central nervous system. While the effects of acute and recurrent stress on key components of the neuroendocrine stress response have been studied, relatively little is known about the consequences of

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chronic stress during early life. Thus, a single acute stress (cold exposure or saline injection) resulted in increased CRH mRNA expression in the 9-day-old rat paraventricular nucleus (PVN), consistent with compensatory upregulation of CRH gene transcription after enhanced secretion of hypothalamic CRH (4, 5). In contrast, repeated acute stress did not increase PVN-CRH mRNA expression (6). Similarly, a longer (24 h) maternal deprivation stress also did not influence CRH gene expression (7); but see Smith *et al*. (8), who reported reduced CRH mRNA expression. These data indicate that the influence of a single or recurrent acute stress, as well as that of more protracted stressful experiences, on molecules involved in the neuroendocrine stress response may be highly variable and thus not predictive of the effects of chronic stress.

The goals of the present study were to gain insight into the short-term effects of chronic psychological stress on the molecules that comprise the HPA axis in immature rats. This information should provide a key to understanding the mechanisms by which chronic early life stress influences the response to subsequent stressors later in life. Therefore, we utilized a paradigm of chronic psychological stress, based on creating an abnormal rearing environment that alters maternal behaviour (9). The overall strategy was to establish the validity of this paradigm as a model of chronic stress, and compare the regulation of key components of the HPA axis in immature (9-day-old) rats subjected to chronic, psychological stress derived from altered cage environment with that of rats reared undisturbed. Specifically, CRH mRNA expression in PVN, the binding capacity and mRNA expression of the CRH receptors CRF_1 and CRF_2 in selected regions, as well as GR mRNA in hippocampus, frontal cortex and PVN, were determined. In addition, parameters indicating the presence of chronic stress, i.e. body and adrenal weights and basal plasma corticosterone (CORT), were examined.

Materials and methods

Animals and chronic stress paradigm

Immature rats of both sexes were offspring of primiparous, timed-pregnancy Sprague-Dawley rats (Zivic-Miller, Zelienople, PA, USA). These were shipped during gestational age 13–15 and maintained in National Institutes of Health (NIH) approved, uncrowded, temperature controlled animal facilities, on a 12-h light/dark cycle with unlimited access to laboratory chow and water. Cages were inspected for presence of pups at 12-h intervals, and the date of birth was considered day 0. Litters were mixed and culled to 12 pups on the first postnatal day (typical litter size in this colony is 12–14 pups) (4, 6, 7, 9–11). Overall, four litters (a total of 48 rats per group) were assigned to each treatment, and 65 brains (derived equally from all litters) were used for the studies reported here (others were perfused or technically unsuitable). All experimental manipulations were conducted at 08.00–10.00 h to minimize potential diurnal variability in HPA-associated gene expression and responses to stress (12). All experiments were carried out according to the NIH guidelines for the care of experimental animals with approval by the Institutional Animal Care Committee.

The comparison (control) group consisted of rats that were considered ‘undisturbed’ (U). After inspection on the first day of life, litters were completely undisturbed (and bedding was not changed) during postnatal days 2–9. Specifically, bedded cages contained approximately 0.33 cubic feet of Sani-Chips (Vermont hardwood). For the chronic stress group, the paradigm described earlier (9) was used. Briefly, cage environment and maternal behaviour were altered by placing pups and dams in cages with limited bedding material (NB) on postnatal day 2. Cages were fitted with small-gauge wire-mesh bottom, raised 2.5 cm from the cage floor (allowing collection of droppings). The sole bedding material consisted of 1–2 paper towels (approximately 0.09 cubic feet). These were shredded by

dams to provide a nest area, and were not replaced. As for the undisturbed (U) group, these rats (U-NB) were not disturbed during postnatal days 2–9.

In this group, the significant reduction of bedding led to diminished ability of the dam to construct a satisfactory nesting area (9). The dams were observed to use the towels for a rudimentary nest, but also exhibited erratic and unusual maternal behaviour. Specifically, they were observed not to attend the pups for prolonged periods that were interspersed with periods of moving the pups individually to other parts of the nest. The dams appeared 'hyper-vigilant' and nervous, but no pup mortality was noted. The pups were repeatedly observed to suckle vigorously, and had no evidence of diminished feeding behaviour; however, they failed to gain weight to the extent of those in the control group.

Tissue harvesting and handling and hormonal assays

On the morning of postnatal day 9, the dams were removed from the cage and the pups were rapidly weighed, and decapitated within 8 min of disturbance. Previous studies had shown that by postnatal day 9, the decreased bedding experience had already led to profound alteration of hormonal stress-responses compared with those of pups reared under a variety of other conditions (4, 6, 7, 9–11). Brains were rapidly dissected onto powdered dry ice and stored at −80°C. Coronal sections (20 μm) spanning prefrontal cortex to ventral hippocampus (9–11) were cut using a cryotome, and every 10th section stained for orientation purposes. Sections were sorted into matched series for *in situ* hybridization histochemistry as described in detail previously (4, 6, 7, 10, 11). The neuroanatomical coordinates (templates) for the analysed regions were determined using a postnatal day 10 rat brain atlas (13), and are described in the analysis section. In addition, pituitaries were collected for binding assays, and adrenals were weighed. Trunk blood was collected for analysis of CORT concentrations using a commercial radio-immunoassay kit (ICN, Irvine, CA, USA) as previously described (4, 6, 9–11). Assay sensitivity was 0.5 μg/dl, intra-assay variability was approximately 5–7%, and interassay variability, measured using three dilutions of adult rat plasma, averaged 10–15%.

In situ hybridization histochemistry (ISH) and probe preparation

ISH and probe labelling were performed as described previously for oligonucleotide probes (4, 6, 7) or cRNA probes (10, 11, 14). Briefly, for CRH mRNA analysis, sections were brought to room temperature, air dried and fixed in fresh 4% buffered paraformaldehyde for 20 min, followed by dehydration and rehydration through graded ethanols. Sections were exposed to 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8) for 8 min and were dehydrated through graded ethanols. Pre-hybridization and hybridization steps were performed in a humidified chamber at 40°C in a solution of 50% formamide, 5 × rSET, 0.2% SDS, 5 × Denhardt's, 0.5 mg/ml salmon sperm DNA, 0.25 mg/ml yeast tRNA, 100 mM dithiothreitol and 10% dextran sulphate. Following a 1-h prehybridization, sections were hybridized overnight with 0.5×10^6 c.p.m. of ^{35}S-labelled oligonucleotide probe complementary to the coding region of CRH mRNA (4). After hybridization, sections were washed, most stringently at 0.3 × SSC. For detection of GR, CRF_1 and CRF_2 mRNAs, sections were hybridized overnight at 55°C with 1×10^6 c.p.m., respectively, ^{35}S labelled ribonucleotide probes complementary to 456 bases of rat GR gene (11, 14), 600 base pair fragment of CRF_1 (15), and 461 base pair fragment of CRF_2 (10, 15, 16). After hybridization, sections were washed in 2 × SSC for 5 min at room temperature and were digested with Rnase (200 μg/ml RNase A; Calbiochem, La Jolla, CA, USA) in a 10-M Tris HCl (pH 8)/NaCl for 30 min at 37°C. Sections underwent serial washes of increasing stringency at 55°C, the most stringent being at 0.03 × SSC for 1 h. For both procedures, sections were then dehydrated through 100% ethanol, air dried and apposed to film (Hyperfilm β-Max, Amersham, Arlington Heights, IL, USA) for 7–14 days.

Pituitary CRH receptor binding

After removal of the pituitaries, the posterior lobes were discarded, and anterior pituitaries were frozen on powdered dry ice and stored at −80°C. Pituitaries from each experimental group (n = 32 each) were pooled (4–8 per group). The tissue was homogenized using 15 strokes of a Teflon pestle homogenizer in 30 volumes of ice-cold buffer (50 mM Tris-HCL, 10 mM $MgCl_2$, 2 mM EGTA pH 7.2). Homogenates were centrifuged twice (50 000 *g* for 30 min at 4°C) in the same buffer, and the pellet was used for the receptor assay. All pellets were resuspended in buffer containing also 0.2% BSA, aprotinin (150 kallikrein units/ml) and 0.1 mM bacitracin, to a final concentration of 80–100 mg wet weight/ml. The radio receptor assay was performed as according to De Souza *et al*. (17). Briefly, 100 µl of the membrane suspension was added to 1.5 ml silanized polypropylene Eppendorf microfuge tubes containing 100 µl of an [^{125}I]-Tyr ovine CRF (oCRH) solution (approximately 40 000–50 000 c.p.m.) and 100 µl of incubation buffer or an appropriate concentration of unlabelled oCRH (Bachem, Torrance, CA, USA) as a displacing agent, for a final volume of 300 µl. Incubation was at room temperature (22°C) for 2 h. The incubation reaction was terminated by centrifugation twice for 7 min each at 12 000 *g*. The resulting pellet was washed gently with 1 ml of ice-cold phosphate-buffered saline (PBS, pH 7.2) containing 0.01% Triton X-100. The supernatant was aspirated and the radioactivity of the pellet was measured in a γ-counter at 80% efficiency. Protein content was determined using a commercial assay (Bio-Rad, Richmond, CA, USA) based on the method of Bradford (18).

Acquisition and quantitative analysis of ISH signal

Semi-quantitative analyses of CRH, CRF_1, CRF_2 and GR mRNAs were performed following *in situ* hybridization without knowledge of treatment, as described in detail previously (4, 6, 10, 11). Briefly, digitized images of each brain section were analysed using the ImageTool software program (University of Texas Health Science Center, San Antonio, TX, USA). Densities were calibrated using ^{14}C standards and are expressed in nCi/g, after correcting for background by subtracting the density of the hybridization signal over the corpus callosum. Coordinates for the structures analysed, with reference to bregma were based on a 10-day-old rat atlas (13). For the hippocampal formation, levels corresponding to 2.9–2.0 mm were used. Because little GR mRNA expression is found in other regions of the hippocampal formation of the 9-day-old rat (11, 19–21), this transcript was analysed only in CA1. CA1, CA3 and the granule cell layer of the dentate gyrus were analysed for CRF_1 mRNA (15, 22). Frontal cortex sections were analysed at coronal levels corresponding to 3.5–2.3 mm. PVN was sampled at levels including the dorsomedial parvocellular cell group expressing CRH and GR mRNA (3.8–3.5 mm) (4, 6, 11), and the ventromedial nucleus (VMH) was analysed at coronal levels corresponding to 3.2–2.3 mm (10, 16).

Statistical analysis

Results are depicted as means with standard errors (SEM). Statistical significance was set at $P < 0.05$. Because most analyses compared two experimental groups, differences were evaluated using unpaired Student's t-test, with Welch's correction for unequal variance when indicated (6, 10, 11). For binding assays, binding curves and receptor affinities and concentrations were analysed by nonlinear regression and a Scatchard plot (Prism GraphPad, San Diego, CA, USA).

Results

Basal plasma CORT concentrations, adrenal weight and animal weight indicate the presence of chronic stress in rats reared with reduced bedding (U-NB)

As shown in Fig. 1(A), basal CORT concentrations were significantly increased in the U-NB group compared to the comparison group (1.90 ± 0.10 versus 1.3 ± 0.05 μg/dl, mean ± SEM). This was consistent with a state of either acute or chronic stress in the former group. However, increased adrenal weight in the U-NB pups compared to the undisturbed group (30.21 ± 0.89 versus 24.25 ± 1.63 mg, $P < 0.01$, mean ± SEM), Fig. 1(B), was highly suggestive of presence of adrenal hypertrophy, a marker of chronic stress. Chronically stressed rats weighed less than the controls (17.74 ± 0.41 versus 21.93 ± 0.35 g, mean ± SEM), although on day 2, prior to the onset of the experiment, weights of control and experimental groups did not differ (8.40 ± 0.163 versus 8.44 ± 0.164 g, respectively, mean ± SEM). In view of the lower weight of the stressed group, the relative increase of adrenal weight, expressed per 100 g body weight (Fig. 1C,D), was even more striking ($P < 0.001$).

CRH receptor binding capacity and mRNA expression are selectively reduced in chronically stressed immature rats

Chronic stress may be associated with increased CRH secretion, resulting in reduction of pituitary CRH receptors (23). Therefore, we compared binding capacity of CRH receptors in pituitaries of the U-NB and U groups. Indeed, as shown in Fig. 2(A), pituitary CRH receptor binding was decreased by 75.63% in the U-NB group at the end of the chronic stress experience. Because recent studies (10, 16) demonstrated downregulation of CRF_2 expression in VMH of immature rats after certain stressful situations (i.e. maternal separation), we measured CRF_2 mRNA in VMH of rats subjected to this chronic stress paradigm. As shown in Fig. 2(B), and unlike the findings after the relatively acute stress of maternal separation, VMH-CRF_2 mRNA levels did not differ between chronically stressed and undisturbed rats (80.59 ± 8.42 versus 90.00 ± 9.86 nCi/g, $P = 0.47$, mean ± SEM).

In the immature rat, CRH binding to its receptor, specifically CRF_1, has been shown to enhance mRNA expression (24). This effect was particularly pronounced in the hippocampal formation. Therefore, we evaluated CRF_1 expression in hippocampus of chronically stressed immature rats in this paradigm. Indeed, CRF_1 mRNA expression in CA1 from U-NB pups was reduced, 62.66 ± 12.36 compared to 116.36 ± 21.92 (mean ± SEM) nCi/g in the controls (Fig. 2C). Expression in dentate gyrus was also lower: 66.70 ± 8.40 versus 123.60 ± 22.90 nci/g in the control group. In contrast, CRF_1 mRNA expression in the CA3 pyramidal cell layer was not affected.

CRH mRNA expression in PVN is significantly reduced in the chronically stressed immature rat

In models of acute stress in both immature and adult rat, increased CRH release leads to upregulation of CRH mRNA in PVN to levels that are higher than those of unstressed controls (4, 6, 25). We questioned whether this enhanced expression occurred also with chronic stress. To our surprise, and as shown in Fig. 3, CRH mRNA expression in the chronically stressed group was significantly decreased, to 66.37% of undisturbed controls (302.00 ± 15.46 nCi/g in U-NB versus 455.00 ± 23.68 nCi/g in U rats, $P < 0.01$, mean ± SEM).

GR gene expression in PVN and frontal cortex, but not hippocampal CA1, is reduced in the chronically stressed immature rat

Glucocorticoids inhibit the HPA axis directly at different sites in the brain, including the PVN (14, 26, 27) hippocampus (28) and frontal cortex (29). Therefore, we analysed GR

mRNA expression in these regions, and found suppressive effects of early life chronic stress on GR mRNA expression in PVN (79.1 ± 8.1 nCi/g versus 135 ± 24.9 nCi/g, mean ± SEM, in stressed and control groups), and in frontal cortex (39.1 ± 2.8 and 50.7 ± 4.0 nCi/g, respectively; Fig. 4A). Chronic stress did not alter GR mRNA expression in hippocampal CA1. Representative PVN sections after *in situ* hybridization for GR mRNA demonstrate a decreased signal over PVN derived from a rat subjected to the chronic stress paradigm (Fig. 4B).

Discussion

The current study demonstrates that altering cage environment via limitation of bedding material leads to significant chronic stress in the immature rat, associated with changes in gene expression at multiple levels of the HPA axis. CRH mRNA expression in PVN was reduced, yet decreased pituitary CRH binding capacity was consistent with enhanced hypothalamic CRH release during this chronic stress. The mechanisms accounting for the reduction of hypothalamic CRH mRNA expression may involve altered glucocorticoid negative feedback, as suggested by the reduced GR mRNA expression in PVN and frontal cortex of chronically stressed pups. Alternatively, or in concert, increased CRH release and a failure of acute stress-induced facilitation of CRH production may contribute to reduced hypothalamic CRH mRNA levels. Taken together, these findings indicate that chronic stress early in life leads to a profound and distinctive alterations of the molecular underpinnings of the neuroendocrine stress response.

This study demonstrated that experimental manipulation of the rearing environment led to changes in the pups that are typical for chronic stress: increased basal CORT secretion (30–32), increased adrenal weight and decreased body weight (30, 31). This chronic stress was provoked by placing pups and dams in cages with limited bedding material. Bedding type and volume are important components of the dams' nesting environment, and limiting the amount of available bedding constituted a continuous stressor for the dam and pups, as well as altered dam–pup interaction (9). Intriguingly, the effects of this significant chronic stress on the molecules governing the central pathways of the stress response: hypothalamic CRH expression, expression and binding of CRH receptors, and glucocorticoid receptor expression in hypothalamus, hippocampus and frontal cortex, were distinguished from those observed in chronic stress in the adult, indicating that the stage of development of the limbic-hypothalamic-pituitary-adrenal axis played a critical role in the processes triggered by this chronic stress.

The neuroendocrine stress response of the immature rat, specifically on days 4–14 of life, is characterized by attenuated hormonal responses and altered gene regulation in response to stress as compared to the adult situation (33–36). This stress hypo-responsiveness during development appears to be stressor-specific, since the HPA axis is fully capable of responding to stimuli that may be considered stressful to a neonatal rat (e.g. cold or saline injection) (4–6, 37). In addition to stress-selectivity, the neuroendocrine stress response during this period is highly regulated by maternal input, involving both feeding and sensory signals (5, 10, 33, 34, 36).

The contribution of developmental stage and maternal regulation to the molecular and hormonal responses to acute stress in the 4–14-day-old rat are currently being elucidated. Thus, maternal deprivation for 24 h enables a higher adrenocorticotropic hormone (ACTH) (38, 39) and CORT (7, 35) response to acute stress. Importantly, the hypothalamic mechanisms effecting stress-induced ACTH release appear to be under maternal regulation. While basal CRH mRNA levels were found to be either unchanged (7) or decreased (8), we (40) and others (5, 38) have shown rapid stress-induced transcription of the CRH gene

throughout the 'stress-hyporesponsive' period in naive rats. Interestingly, at early ages (e.g. P6), no changes in CRH mRNA were found at 4 h after stress (4, 5, 38), although an increase was reported at the 15-min time-point (5, 38). In contrast to the response to acute stress under 'normal' maternal–pup interaction, maternal deprivation led to augmented stress-induced synthesis of vasopressin in the 12-day-old pup, which may have contributed to the larger ACTH secretion (38).

The molecular basis of the maternally dependent stress-induced transcription of hypothalamic CRH gene may involve glucocorticoid receptor activation in hypothalamus, frontal cortex and hippocampus. Indeed, reduced GR mRNA in PVN, hippocampal CA1 and frontal cortex have been reported after maternal deprivation, with likely consequences on the magnitude of negative-feedback modulation of CRH expression (20, 39). Thus, elements of maternal input appear to be critical to both the molecular and hormonal neuroendocrine responses of the immature rat to acute stress.

Whereas the unique, age-dependent molecular mechanisms of the response of the immature rat to acute stress have been emerging, relatively little insight has been gained into mechanisms of the molecular and hormonal response to chronic stress. It is logical to consider that these mechanisms would include both processes that govern the mature HPA axis, as well as age-specific (and potentially maternal dependent) molecular cascades. In the adult rat, chronic stress has been shown to affect CRH mRNA expression in a complex manner, and increased (41–43), decreased (31, 32, 44) or unchanged (45, 46) levels have been found, depending on the stress paradigm. In the context of the current study, chronic or intermittent stress in mature rats was found to result in relative desensitization of the CRH-neuroendocrine system, and increase of the role of vasopressin, synthesized in the parvocellular PVN, as an activator of the HPA axis (46–48). Furthermore, vasopressin-induced CORT secretion has been shown to elicit downregulation of CRH mRNA expression in PVN (as found in the current study in the immature rat). In the adult, however, this enhanced role of vasopressin required at least 2 weeks of chronic or intermittent stress (31, 46, 47). In this respect, the recently described rapid enhancement of vasopressin expression in immature rat PVN upon acute stress in the setting of maternal separation is intriguing (38). These data indicate that the onset of modulation of vasopressin expression (and consequent contribution of this hormone to the stress response) may be significantly faster during the second week of postnatal life (49).

Indeed, the remarkably rapid reduction of CRH mRNA levels in PVN of the chronically stressed pups in the current study, evident already by P9 (i.e. after 1 week of the modified rearing conditions), is consistent with a more rapid time-course of the adaption to chronic stress, which may be specific to this age (49). Several processes may be involved in this suppression of CRH gene expression. First, the reduced CRH mRNA levels occurred in the face of chronically elevated glucocorticoid levels, as has been found also by area-under-the curve analysis in our previous study (9). These findings are consistent with a negative feedback evoked by the stress-associated chronically elevated plasma glucocorticoid concentrations (39). Glucocorticoids have been shown to depress hypothalamic CRH mRNA expression in adult PVN (26, 27), but earlier studies suggested a relative lack of glucocorticoid negative feedback on PVN-CRH expression in rats younger than 9 days (14, 39). However, more recent studies support the presence of a robust glucocorticoid-induced negative feedback in the 9-day-old rat, particularly when glucocorticoid levels are elevated persistently (6, 39). Thus, a reasonable explanation for the reduced hypothalamic CRH mRNA levels may derive from a glucocorticoid negative feedback.

It should be noted that the decreased GR mRNA expression in PVN of chronically stressed rats in the current study (Fig. 3), might predict reduced transduction of the glucocorticoid

signal onto CRH-producing PVN neurones. However, GR mRNA levels were analysed at the termination of the chronic stress, and their reduction may therefore be a consequence of the chronically elevated glucocorticoid plasma levels, rather than a reflection of the acute, early events induced by the application of the chronic stress paradigm in the immature pup brain. In addition, the net effects of these region-specific changes in GR expression would further influence the magnitude of the neuroendocrine response to future stresses. The reduced cortical GR mRNA expression [consistent with the implication of cortical-GR inhibitory signalling onto the HPA axis response to stress (29)], would be expected to lead to enhanced HPA reactivity to subsequent stressors (23).

A second possible mechanism for the reduced CRH mRNA in PVN may result from persistent increased CRH release, without compensatory enhanced synthesis, leading to depleted mRNA stores (6, 45). Increased CRH release is suggested from the large (76%) reduction in pituitary CRH receptor binding, consistent with receptor desensitization by enhanced secretion of hypothalamic CRH, although an alternative possibility, that absence of the 'trophic' effect of CRH may reduce corticotroph receptor synthesis, cannot be excluded. Chronic stressors have been found to downregulate pituitary CRH receptors in adult rat (32), and potential mechanisms may include internalization and thus reduction of receptor binding capacity resulting directly from CRH binding, as shown after infusion of CRH intermittently for 3 days (50) or continuously for 48–50 h (51). Alternatively, chronic stress-related increase of plasma glucocorticoids may affect pituitary CRH receptors: chronic CORT decreased the relative density of CRH receptors in corticotrophs *in vitro*, by increasing receptor internalization rate (52). In the immature rat, pituitary CRH receptors may be even more sensitive to circulating glucocorticoids (53). Thus, the reduced CRH receptor binding found here may derive from both increased CRH secretion and high glucocorticoid levels, both of which may also contribute to the observed reduction of hypothalamic CRH mRNA levels.

Thus, a dynamic chain of events may be provoked by the persistent stress imposed on 2-day-old rats, resulting in the spectrum of molecular changes of the HPA axis noted above. A schematic presentation of this putative sequence of events is depicted in Fig. 5. In this scenario, bedding limitation initially acts as an acute stress, activating the immature rat's HPA axis in the context of abnormal maternal interaction. Acutely, CRH release (4, 6) and enhanced 'compensatory' transcription occur, leading to elevated plasma stress hormones. However, during the first few postnatal days, CRH transcription does not result in persistent elevation of CRH mRNA expression (4, 5, 40). Indeed, CRH transcription and steady-state mRNA levels may be further downregulated by the chronic, GR mediated negative feedback resulting from elevated plasma steroids. By day 9, CRH mRNA levels are reduced, and the persistently elevated plasma CORT levels are driven also by other ACTH secretagogues, most likely vasopressin (38, 46, 47). These chronically high CORT levels eventually downregulate GR mRNA in regions involved in HPA and hypothalamic CRH mRNA regulation (frontal cortex and the PVN itself) but, by that time, a new steady-state has been reached (vide infra).

In this new steady state, hypothalamic neuroendocrine functions are governed by altered 'higher' limbic structures and pathways. This is evident not only from the selective reduction of GR mRNA, noted above, but also from the remarkable reduction of the expression of the stress-mediating CRH receptor, CRF_1, in hippocampal CA1 and dentate gyrus. It might be noted that in adult studies of chronic stress, hippocampal CRF_1 mRNA expression was found to increase (54), decrease (55) or remain unchanged (50) depending on the stress paradigm. In the context of the current study, CRF_1 binding capacity and CRF_1 mRNA expression in the immature rat have been shown to be regulated by CRH itself (24), so that reduced CRF_1 mRNA expression in hippocampus here is consistent with reduced

local hippocampal CRH. While the current study did not examine CRH levels in this region, other work suggests that, during the developmental age discussed in this study, hippocampal CRH expression is at least twice as high as in mature hippocampus (56, 57), and may be regulated by selective acute stressors (58). Whether hippocampal CRH expression is regulated by chronic stress, such as the current paradigm, and influences CRF_1 mRNA levels, requires further study.

In summary, chronic stress resulting from alteration of cage environment leads to profound alterations of the molecular effectors of the neuroendocrine stress response at multiple hierarchial levels. These include CRH mRNA expression in PVN, binding capacity and mRNA concentrations of CRH receptors, and glucocorticoid receptor (GR) mRNA expression in central nervous system regions involved in the regulation of the neuroendocrine stress response. These findings may pertain to the human situation, where early life stress has been shown to lead to persistent alterations of HPA axis function including elevated basal glucocorticoid concentrations, impaired glucocorticoid feedback, and alterations in CRH-receptor regulation, not unlike those found here. Therefore, this model may provide a useful tool for understanding the molecular mechanisms involved in the reciprocal interactions of early life chronic stress and the HPA axis, and of the long-term alterations of the neuroendocrine stress response induced by early life stress.

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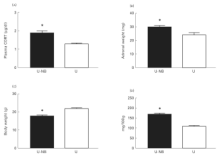

FIG. 1.

Indicators of the induction of chronic stress by experimental manipulation of the rearing environment of neonatal rats. (A) Basal plasma corticosterone (CORT) concentrations were significantly increased in the group reared with limited cage-bedding (U-NB, n = 11 animals per group) compared to the 'normally' reared undisturbed controls (U; n = 12). (B) A more specific marker of *chronic* stress, increased adrenal weight, was found in the experimental group (n = 12 per group). (C) Chronic stress was also evident from modest, but significant, reduction in body weight in the experimental group (n = 48 per group). (D) Adrenal weight expressed per 100 g body weight better reflects the significant hyperplasia of adrenal tissue associated with the chronic-stress in the U-NB group ($P < 0.01$). All parameters were measured on postnatal day 9, at the termination of the experiment. Values are expressed as means ± SEM. *$P < 0.05$.

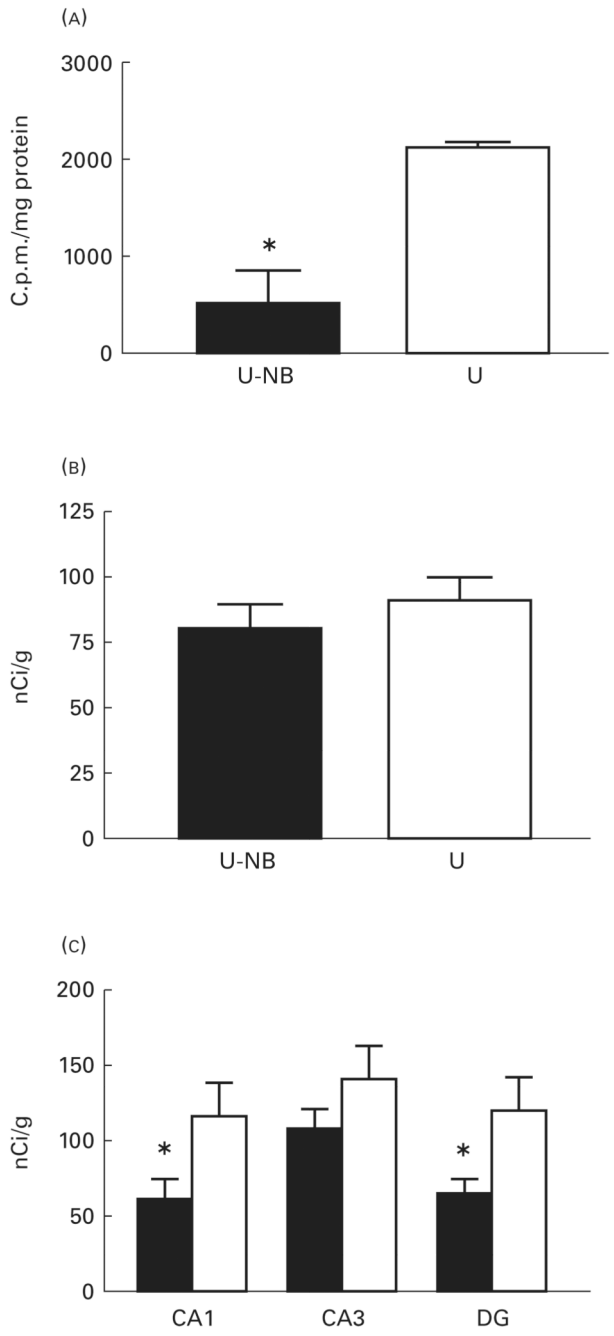

FIG. 2.

(A) Pituitary corticotropin releasing hormone (CRH) receptor binding capacity was decreased by 75.63% in chronically stressed (U-NB) immature rats. Binding capacity was determined in pituitary homogenates as described in the Methods section, using pooled (4–8 per tube) pituitaries from 32 rats per group, and includes both receptor types. (B) mRNA concentration of the CRF_2 receptor in the hypothalamic ventromedial nucleus (VMH), shown to be influenced by some subacute stressors in the immature rat (10, 16), did not differ between chronically stressed (U-NB) and undisturbed rats (U; n = 6 per group). (C) mRNA expression of the other member of the CRH receptor family, CRF_1, was reduced in discrete hippocampal fields [CA1 and dentate gyrus (DG)] of the experimental (U-NB)

pups, compared to the controls (n = 10 per group). No change was evident in CRF_1 mRNA expression in the CA3. Values are expressed as means ± SEM. *P < 0.05.

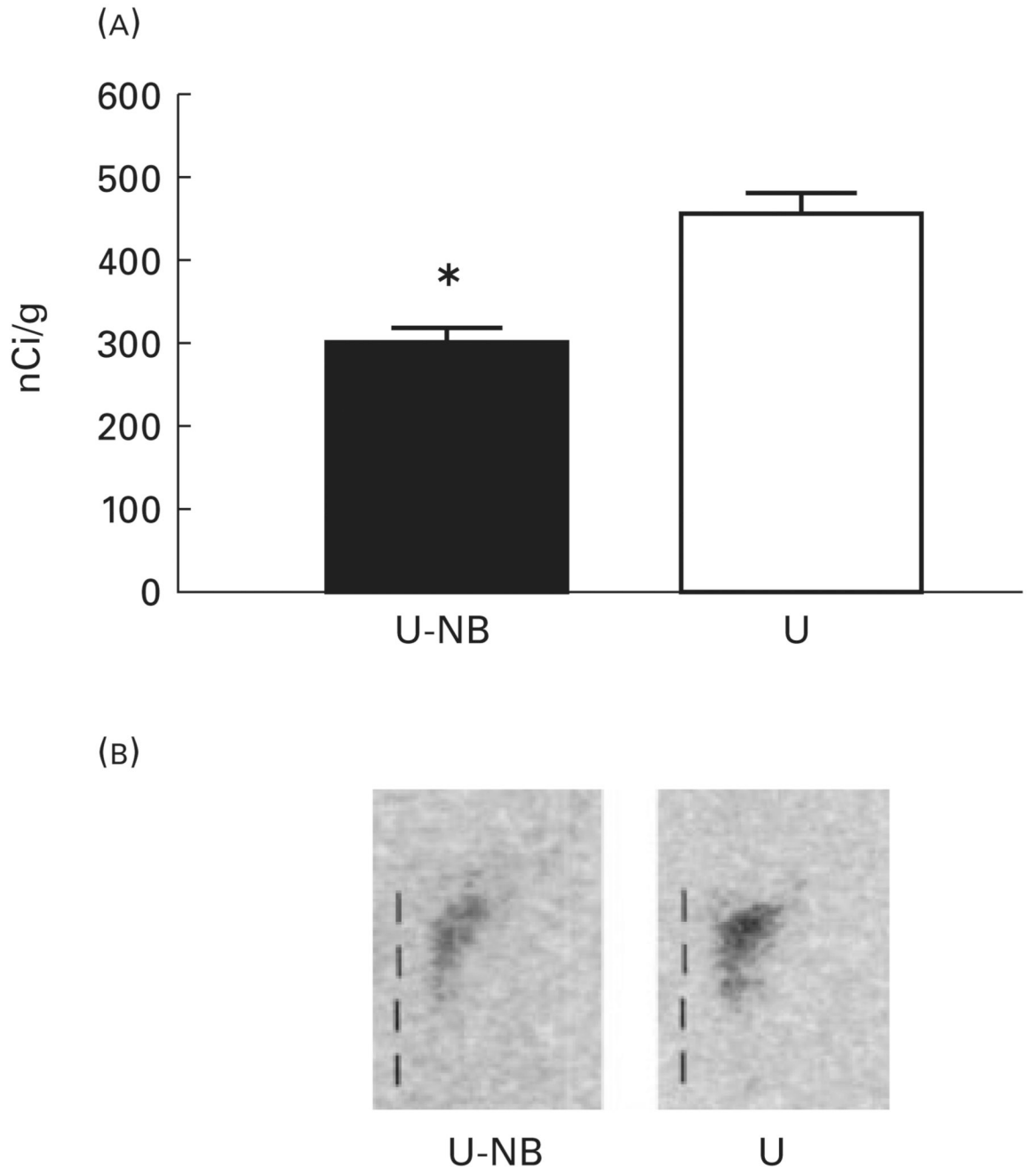

FIG. 3.

Corticotropin releasing hormone (CRH) mRNA expression in paraventricular nucleus (PVN) of 9-day-old rats that had experienced chronic stress during postnatal days 2–9 (U-NB) was reduced compared to that of the undisturbed (U) group (n = 12 animals per group). (A) Semi-quantitative analysis of optical density signal over PVN was performed after *in situ* hybridization of matched coronal sections using unbiased techniques, as described in the Methods section. Values are expressed as means ± SEM. *P < 0.05. (B) Decreased CRH mRNA signal over PVN is evident in a photomicrograph derived from a chronically stressed rat, compared with a matched section from a control.

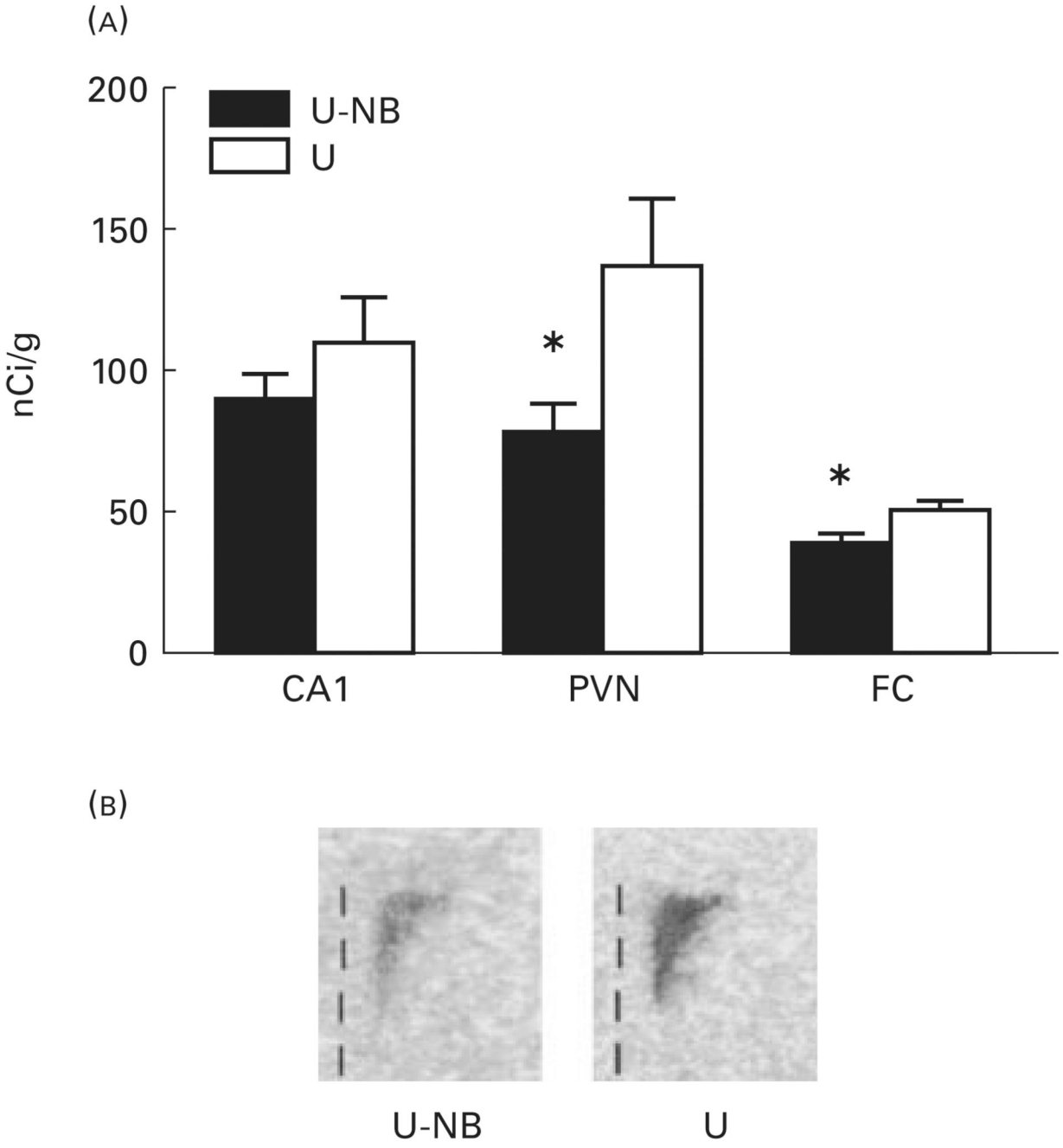

FIG. 4.

Glucocorticoid receptor (GR) gene expression in paraventricular nucleus (PVN) and frontal cortex (FC), but not in hippocampal CA1, were reduced by early life chronic stress (n = 12 per group). (A) Semi-quantitative analysis was performed on sections subjected to *in situ* hybridization for GR mRNA. Significantly (*) lower GR mRNA signal is evident over PVN and FC of the experimental group (U-NB) compared with the undisturbed controls (U). Values are expressed as means ± SEM. (B) Representative sections of the PVN illustrate the quantitative data shown in (A).

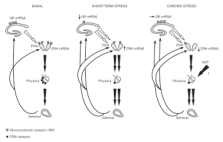

FIG. 5.

Schematic presentation of the putative changes in gene expression and hormonal release patterns during the evolution of the chronic stress state in the neonatal rat. The normal steady-state is shown on the left. The middle panel depicts changes induced acutely by combined physical/psychological stress (4, 6, 7, 10, 11, 40). Increased corticotropin releasing hormone (CRH) release and compensatory enhancement of CRH expression result in increased CRH-induced adrenocorticotropic hormone (ACTH) and corticosterone (CORT) production. The elevated plasma CORT levels result in downregulation of glucocorticoid receptor (GR) mRNA in hippocampus and paraventricular nucleus (PVN). The panel on the right demonstrates the achievement of a new chronic stress 'steady-state'. In this state, ACTH and CORT production and release is likely driven not only by the depleted hypothalamic CRH (the functions of which may be further minimized by reduced pituitary CRH receptors), but potentially also by vasopressin (AVP) or other secretagogues. The augmented drive of the adrenals leads to their hypertrophy, and to chronically high plasma glucocorticoid levels. These downregulate GR mRNA expression in frontal cortex (not shown) and PVN, and contribute to the depression of hypothalamic CRH expression. Filled arrows denote a positive, facilitatory action whereas open arrows denote negative influences.